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CONTRACTING ORGANIZATION: Arizona State University
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Tempe, AZ 85287-6011

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SEE BELOW

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Abstract: We invented the immunosignature technology (IMS) as a simple, universal diagnostic. Our goal has been to apply it to the early detection of disease through simple health monitoring. In the project funded here we proposed to determine if IMS could be applied to the early detection of breast cancer. It is well established that detection of breast cancer at Stage I or earlier has a 100% 5 year survival rate. We proposed to use the prospectively collected, NCI-PLCO samples for this project. 150,000 people were enrolled over 8 years (1993-2001) and half were screened for prostrate, lung, colon and ovarian cancer over. Later standard clinical information on breast cancer was linked to these samples. Our plan was to first test samples collected 0-1 year before diagnosis of Stage I bc. If this were successful the study would be extended to samples collected even earlier before diagnosis. A second aim was to determine if IMS could be applied to DCIS and benign cancer. A third would be to follow the time course of cancer in 30 of the PLCO subjects. In our first year almost all the effort was aimed at determining an IMS in the 0-1yr PLCO samples. We could not do this. This was reported to the DoD panel. We assumed the samples were good and that it was the fault of the quality of the arrays we were using. The panel suspended funding and requested that we demonstrate the array quality was good and that we complete the analysis of the 0-1year samples from PLCO before funding was resumed. In the intervening 5 months we have made great progress on the array quality, as we report here. However, the inability to determine an IMS in the PLCO samples was because of poor sample quality as we also demonstrate. We demonstrate on different sample sets both Stage I bc and DCIS have distinct signatures from women without bc. We also demonstrate that samples collected 0-18m before diagnosis of Stage I melanoma cancer in the UKCTOCS program, which was done approximately at the same time and protocol as the PLCO, provide a clear IMS of early melanoma. We also show by objective criteria that the PLCO samples are very different than the samples from UKCTOCS (prospective collection of 150,000 women) and from the CTS blood donation center. We now propose to use IMS to establish the signature of Stage I and DCIS. We will use well-qualified samples from the Duke Medical Center and UKCTOCS. Additionally, we will compare the Stage I signature from samples from the US, England and Russia to determine the variance in the same cancer across regions. We have initiated discussions with Abcodia, Inc and HealthTell, Inc for the development and commercialization of a diagnostic for Stage 1 breast cancer if we are successful.

TABLE OF CONTENTS

1. INTRODUCTION:

We want to develop a simple, inexpensive and accurate method to detect breast cancer (bc) early. We propose to test whether the immunosignature (IMS) technology can detect bc early. We were going to use the PLCO samples to test this. However, we have now demonstrated that these samples are defective. We propose modified aims to determine, using other sample sources that we have tested, if there is a robust IMS for Stage I cancer and DCIS. We will also determine if the IMS of Stage I is the same in three different parts of the world. If successful, women could monitor for early signs of bc by simply sending in a drop of blood.

2. KEYWORDS:

Immunosignature, diagnosis of breast cancer, early diagnosis, stage I, DCIS, serology, informatics, immunology,

3. ACCOMPLISHMENTS:

What were the major goals of the project?

1. Determine how early IMS can detect breast cancer. *This could not be accomplished because of the defective PLCO samples. We have demonstrated in small studies that Stage 1 and DCIS have distinct signatures* (10%).
2. Determine if immunosignatures can distinguish benign growths from invasive tumors and distinguish classes of benign growths. *Using Duke samples we have preliminary data this can be done (50%).*
3. Determine the value of personal baseline immunosignatures in detecting cancer early. *This cannot be accomplished now because of the problem with the PLCO samples.*

What was accomplished under these goals?

In the time since the last report we have focused on two goals. First, as requested by the Panel, to demonstrate that the array format was stable could perform IMS effectively. We assumed the problem in demonstrating a signature of the pre-Stage 1 bc was because of array stability and performance. We had previously shown that stage II, III and IV could readily be distinguished from women without cancer and, in preliminary experiments, that DCIS had a distinct signature. However these were smaller studies than we were attempting to do with the PLCO samples. Over the last year we have implemented the following improvements to the arrays:

1. The surface of the array is now coated with epoxy rather than aptes. This allows much more even coating and therefore reproducible peptide density. Reproducible peptide density on each feature across wafer production is the key for stable array production.
2. We invented a method (patent pending) for increasing the length of the peptides from 12aa on average to all being 17aa long, without adding significantly to the time or cost of synthesis. Longer peptides have more discrimination power. This was accomplished by inserting a set of amino acids every 5^{th} position.
3. We test 4 different sets of 5 aa combinations to arrive at a set that had the optimal performance.

In conclusion we have developed a stable high performance standard synthesis. The performance characteristics of our current, CIM 7 arrays, are given in the Table below.

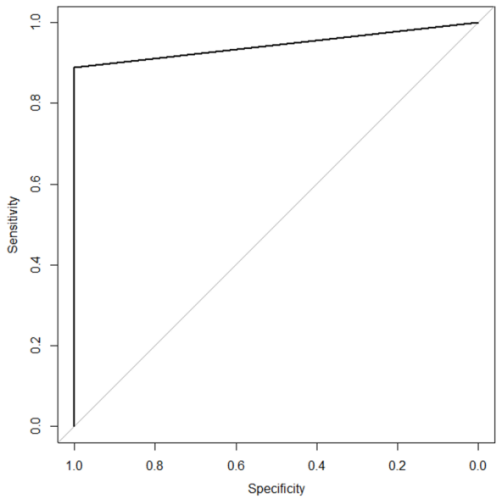
Table 1: Analysis of quality/performance for current and previous generation immunosignature arrays. The previous generation immunosignature microarrays ("Previous gen") were used at the start of this grant. The next generation ("Current gen") encompassed numerous manufacturing improvements. Below are several pertinent comparisons across the microarray generations. For the column "Reproducibility all peptides", the CV is the coefficient of variation, and is the average CV of all peptides across technical replicates either within a wafer ("within") or across multiple wafers manufactured at different times ("across"). 95th %ile fold-change is the sensitivity of detecting a change in a patient given the variability in the arrays. Current gen arrays can detect a <11% change in antibody number on average using 2 technical replicates. Overlapping features defines the number of identical peptides found in a t-test across multiple wafers, signifying the stability of peptide synthesis.

	Peptide length	Total # peptides	Reproducibility all peptides	95th %ile fold-change	Overlapping features Chagas vs. normals
Current gen 125K, 18um square spots	17	122927	CV_{within}=9.1% CV_{across}=12%	1.19_{within} 1.48_{across}	1:2 898/1000 2:3: 750/1000 1:3: 768/1000
Previous gen 330K, 8um round spots	11.2 avg	329219	CV_{within}=21% CV_{across}=110%	1.35_{within} 3.30_{across}	1:2: 0/1000 1:3: 0/1000 2:3: 0/1000

These arrays have been produced by a largely manual method up to now. We recently purchased a custom-built, hands-off automated wafer synthesis system that should improve the synthesis reproducibility even more and more than double synthesis capacity.

Importantly, the current generation IMS arrays have been shown in other diagnostic applications to have unique capability. In projects with collaborators over the last 6 months we have shown:

1. Distinction of people with and without Chronic Fatigue Syndrome. This had only been done before using an extensive panel of immune markers and this assay was only accurate soon after onset of disease.
2. Establish a signature for response to rituximab treatment for Chronic Fatigue Syndrome. This would be a first. IMS is being implemented in new Phase III clinical trials.
3. Distinguish infections by the two species of *Coccidioides*, the causal agent of Valley Fever.
   This has not been done serologically before.
4. IMS distinguished two types of closely related *Rickettsia* infections. This has not been done serologically before.
5. A pilot study distinguished 4 clinical and molecular subtypes of pediatric brain cancer.
6. Serologically distinction of West Nile Virus infection from Dengue infection. The current FDA approved diagnostic for Dengue infection cannot make this distinction.
7. Some capability of distinguishing the 4 Dengue serotypes. Dengue virus has 4 serotypes that have not been distinguished serologically. Using 50 samples of each serotype we demonstrate that IMS has some, but not perfect, discriminatory power. This example may demonstrate the limit of IMS at least on these arrays. But this limit is beyond current standards for this assay.



Accuracy : 0.72
95% CI : (0.5061, 0.8793)
Statistics by Class:

Class:	DEN1	DEN2	DEN3	DEN4	ND
Sensitivity	0.8000	1.0000	0.8000	0.4000	0.6000
Specificity	1.0000	0.8000	0.8500	1.0000	1.0000
PPV	1.0000	0.5556	0.5714	1.0000	1.0000
NPV	0.9524	1.0000	0.9444	0.8696	0.9091
Balanced Acc	0.9000	0.9000	0.8250	0.7000	0.8000

Figure 1: Classification of Dengue serotypes (DENV1-DENV4) Left – AUC for Dengue serotype classification; right – statistical performance figures for Dengue classification.

The point of this list, which only includes exceptional diagnostic applications, is that the current generation IMS arrays are performing very well. This is relevant to the performance we report on the PLCO samples below.

The second request of the Panel was, once the arrays were stabilized, to complete the analysis proposed on the PLCO samples. We will not be able to do this because, as we demonstrate, these samples are defective. However, we will propose a new set of aims that will produce an equally effective advance in diagnosis of breast cancer.

The case for the PLCO samples be defective is made below:

1. Failure to develop a signature on the PLCO pre-stage I samples. We have assayed all 240 cases and 700 non-cancer controls on 9 different types of arrays. A total of 6932 330K IMS arrays were used, 664 10K arrays, and 2232 current generation 125K arrays were used. These arrays all performed well in other diseases studies including other cancer cohorts. However, we could never do better than 50% accuracy in training and calling blinded PLCO samples. We have never experienced this poor performance in over 40 different types of diagnostic projects.

2. 148 case and 148 controls of the PLCO samples were assayed on the arrays. Using this as a training set, prediction of 15 case and 15 controls that were left out of the training was 51% (Figure 2). Results strongly suggest that samples could be the source of the poor performance.

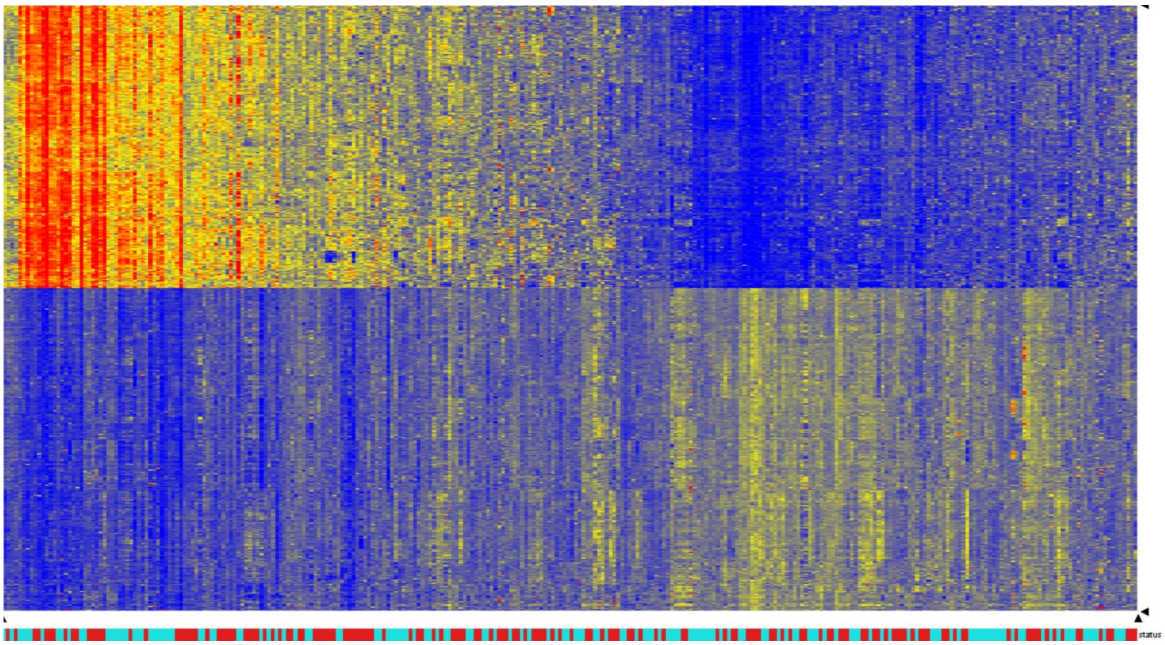


Figure 2: Final attempt to classify PLCO samples - The heatmap show above represents 148 case (samples taken from women diagnosed with breast cancer between 0-1 year later) and 148 age-matched controls collected by the PLCO Clinical Trial. Samples were processed in triplicate on current-generation immunosignature arrays. A t-test between case and control yielded 200 peptides (shown on the Y-axis) that should have identified disease-specific patterns. However, the colored bar shows the mix between case (cyan) and control (red), indicating that there are no immunological signals that correlate to disease. There was only ~ 10x difference between the p-values of the training set peptides and peptides randomly chosen.

3. Successful classification of Stage 1 bc samples. We received 81 Stage I cases (all receptor classifications) and 107 controls from the Duke repository managed by Dr. Jeffery Marks. We assayed them on the same arrays as used in point 2 above for the PLCO samples. The training signature was between 92%-95% accurate with leave 10% out and exhaustive retesting (Figure 3)

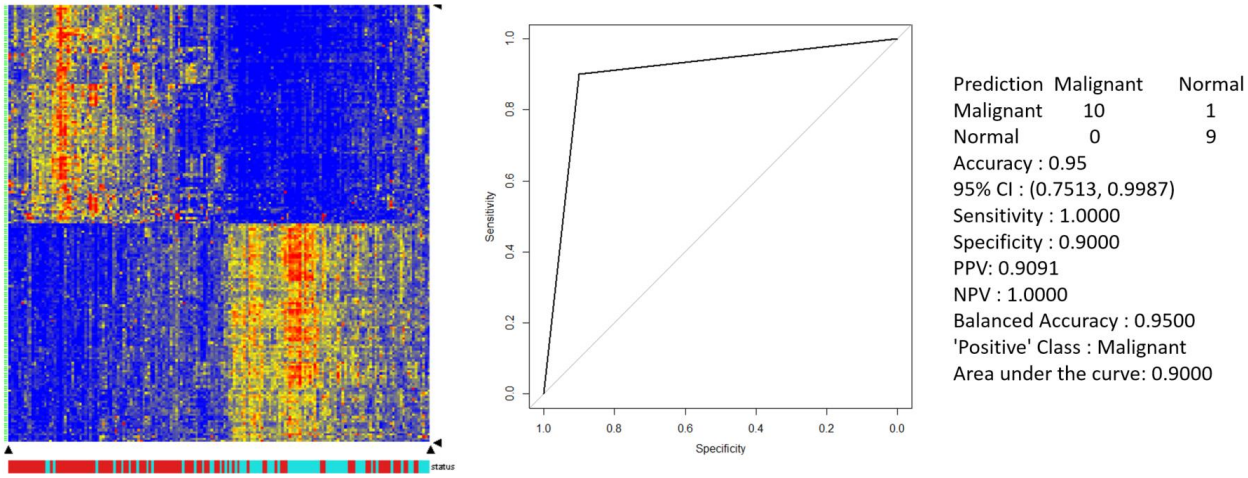


Figure 3: Classification of Stage 1 breast cancer vs. matched controls 104 Stage 1 breast cancer samples were compared to 107 non-cancer female donors (samples received from Duke University, Dr. Jeffrey Marks). Classification performance of held-out samples averaged 0.92. Heatmap on left shows case (red) and control (cyan). AUC for ROC curve (middle) is 0.90. Other performance statistics are shown to the right. There was ~ 10^{-20} fold difference in p-values between the peptides selected from actual case and control vs. selected from shuffled labels.

4. Successful classification of pre-diagnostic melanoma samples. We purchased from Abcodia 84 samples taken 1-18 months before diagnosis of Stage 1 melanoma, and 84 normal, matched controls. This work was not funded by the DoD grant, but we think this analysis is relevant because the samples are from the UKCTOCS prospective collection done for ~150,000 women at about the same time and same protocol as used for the PLCO study. The training set had 84% accuracy with leave 10% out recursive testing (Figure 4).

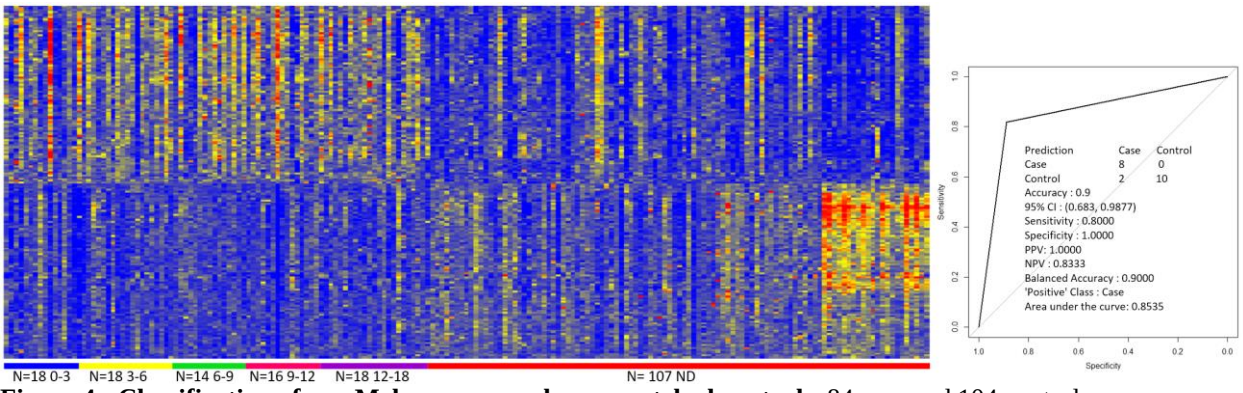


Figure 4: Classification of pre-Melanoma samples vs. matched controls 84 case and 104 controls were analyzed for classification of disease. N=18 samples 0-3 month prior, N=18 three to six, N=14 six to nine, N=16 nine to twelve, and N=18 samples twelve to eighteen months prior to diagnosis of melanoma were compared to 107 age-matched non-cancer samples. Leave 1-from-each-class-out 100-fold cross-validation yielded an average of 84% accuracy, with 96% specificity and 79% sensitivity. It should be noted that it is unknown whether any tumor or pre-malignancy was present at pre-diagnosis. AUC for ROC curve is on average 0.8380. Performance statistics for a single-fold cross validation are shown to the right. There was ~10^{-14} fold difference in p-values between the peptides in the training set and those chosen with shuffled labels.

5. PLCO samples are outliers compared to other samples. On the same CIM 7 arrays we compared the PLCO cases, PLCO controls, UKCTOCS controls, Duke controls and freshly collected blood donation samples from CTS. As can be seen in Figure 5, the PLCO samples are extremely different from the 3 other sources. There is something inherently different about the PLCO samples that is biologically based.

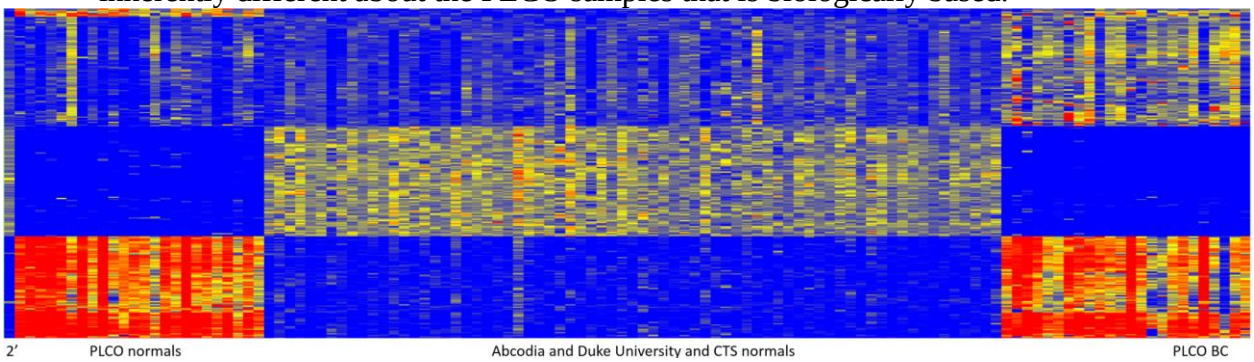


Figure 5: Comparison of samples from PLCO with samples from Abcodia and from Duke University Sera from PLCO, Duke and Abcodia were processed on the same arrays at the same time. The data distribution shape, skewness, kurtosis, and other measures of normality suggest that the PLCO samples originate from a unique source. Samples from both Abcodia (UK) and Duke (US) were indistinguishable, suggesting they are similar to each other.

Unfortunately, we conclude the PLCO samples are worthless relative to IMS at least. We have informed NCI-PLCO and provided this data. We have spent considerable amount of DoD funding (and ~$400K CIM funds) and our time assuming these samples were well handled. PLCO did inform us that we were the first ones to use these bc samples. However, given the PLCO protocol our assessment probably applies to the whole PLCO collection. We point out that we are not the only ones now noting problems with these samples (Zhu *et al*., 2014, see attached). PLCO hypothesizes that the problems may arise from improper mixing before aliquoting.

The mistake I made was to take so long to question the quality of the samples.

On the positive side we have accomplished:

1. Greatly improved the reproducibility and discrimination power of the arrays.
2. Invented a method to increase the length of the peptides at low cost and time
3. Demonstrated that establishing a Stage 1 bc signature is feasible.
4. Demonstrated that establishing a DCIS signature is feasible.
5. Identified and qualified sample sources for proceeding with this project.

What opportunities for training and professional development has the project provided?

Four CIM faculty, 1 graduate student and 5 undergraduate students have been involved in this project and the training that resulted. The focus was solving the problem of lack of performance on the PLCO samples. 4 faculty engaged in how to improve the chemistry of the array development. 3 faculty and a graduate student engaged in improving the analytical and statistical analysis of the PLCO analysis. Undergraduate students were involved in both aspects. All of CIM was kept updated on the approaches and progress or lack of. The most important training for the students was real experience in trouble-shooting large problems. I hope they learned from my mistakes.

How were the results disseminated to communities of interest?

Nothing to report

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Please see "Proposed Next Steps" under Changes/Problems below.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

1. A new wafer surface was developed that greatly improved wafer to wafer performance
2. A new technique was invented to extend the length of the peptides without significantly increasing the number of masks or steps.

What was the impact on other disciplines?

.

The improvements to the surface of the wafers and the length of the peptides will be widely adopted for the use of IMS.

What was the impact on technology transfer?

The improvements to the wafers has been adopted by HealthTell, inc under a sublicense.

The invention of the method to increase length of the peptides was filed on as patent and will likely be licensed by HealthTell and used by others making peptide arrays.

What was the impact on society beyond science and technology?

There are no biomarkers for the early detection of breast cancer. If this project is successful it would open this field. It could enable the implementation of early detection and treatment of cancer with wide reaching implications. It could contribute to the effective elimination of breast cancer.

5. CHANGES/PROBLEMS:

Problems: Our major problem for completing Aim 1 and Aim 3 of the proposal is that the PLCO samples are compromised.

Changes: With the high performance arrays we have demonstrated and the samples we have now qualified, we are confident we can demonstrate for the first time a simple diagnostic for the early detection of breast cancer. No one has been able to do this to date. If we can de-risk this concept through accomplishing the following specific aims, we are also confident that this will lead to the commercial development of the assay. We have already participated in negotiations with HealthTell and Abcodia for this purpose. We can accomplish these aims in less than one year.

Proposed Next Steps

Though we cannot use the PLCO samples, we see a clear path to demonstrate the feasibility of a Stage 1 cancer diagnostic by IMS. As there is no such biomarker, this would be an advance in breast cancer diagnostics. If every woman could simply send in a drop of blood to detect DCIS or Stage 1 cancer it could offer a path to effectively end breast cancer.

Toward this goal we propose accomplishing the following Aims in the next year.

Aim 1. **Demonstrate an IMS for Stage 1 breast cancer**. We will use 100 samples from the Duke repository from women diagnosed with Stage 1 breast cancer and non-cancer controls to establish the training IMS. These samples will represent the normal distribution of receptor/Her2 status, though we have not seen to date any IMS stratification by these factors. This signature will be applied to 50, blinded case and control samples to establish accuracy. We will also apply the same strategy to 100 case (Stage 1) and control samples from Abcodia (UKCTOCS study). This training signature will be applied to the blinded samples. The issue is whether there is a Stage 1 signature, what is its accuracy and does the same signature apply to two sources. These samples are available with clinical information (see Letters attached).

Aim 2. Demonstrate an IMS for DCIS. The same strategy as described for Aim 1 will be applied to DCIS samples from the Duke repository. The same issues apply. In addition, we will determine the extent of overlap between the DCIS and Stage 1 signatures. Our hypothesis is that they will have limited overlap based on the idea that the conversion to an invasive phenotype will generate new antigenic recognitions by the immune system.

Aim 3. **Determine if the IMS for Stage 1 cancer can apply to prospective samples collected in Russia.** In 2013 CIM helped establish an IMS core (Russian American Anti-Cancer Center, RAACC) in Barnaul, Russia. It is associated with the Altai Regional Cancer Center. They are collecting samples to our SOP in the Cancer Center from women diagnosed with breast cancer. The RAACC will use 100 samples from Stage 1 diagnoses and controls from their collection to establish an IMS. This will be tested on 50 case, controls of blinded samples. CIM will send 100 case and controls from the US to be assayed with the RAACC IMS (samples cannot be sent

out of Russia). The accuracy of the RAACC signature to predict US samples will be determined. This will allow us to attribute the contribution of technical differences in running the assays. We will also determine the correspondence of the IMS RAACC establishes on the US samples compared to that determined at CIM.

All the samples for these experiments already exist in the Duke, Abcodia and RAACC repositories (see accompanying letters). We have tested samples from Duke and Abcodia and they are of good performance (Figures 3-5).

Conclusions: At the conclusion of this proposed effort (< 1 yr.) we will have established 1) a signature for Stage 1 cancer tested on samples from three locations, 2) a signature for DCIS compared to Stage 1, and 3) the accuracy of IMS performed at a second site.

We think the successful completion of this work will advance its commercial development. It will de-risk the validation studies that Abcodia and HealthTell would need to complete by showing that the samples for such studies exist and that IMS is feasible. In another proposed study with Dr. Marks we will test the use of blood spot cards for assessing Stage 1 and DCIS diagnosis. We are confident that this work will stimulate commercial development of an IMS for Stage 1 and DCIS breast cancer in the US. It can be directly applied in the Altai Regional Cancer Center.

Changes that had a significant impact on expenditures

The problems with determining that the PLCO samples were compromised took considerably more time and cost than anticipated. Finding and validating other samples was an unanticipated effort and cost. However, we are confident we can complete the modified aims proposed with the remaining funds. .

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications.

Brian O'Donnell, Alexander Maurer, Antonia Papandreou-Suppappola and Phillip Stafford, "Time-Frequency Analysis of Peptide Microarray Data: Application to Brain Cancer Immunosignatures*", Cancer Informatics* 2015: **2** 219-233 *DOI: 10.4137/CIN.S17285*

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Developed new wafer surface. This was communicated to HealthTell under sublicense.

Invented new method to lengthen peptides. Patent filed.

- **Inventions, patent applications, and/or licenses**

Disclosed and filed through ASU on method to lengthen peptides.

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Stephen Johnston No change
Name:	Neal Woodbury No change
Name:	Phillip Stafford No change
Name:	Jeffrey Marks No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Continuing
DHS Contract HSHQDC-15-C-B0008 Beginning 5/1/2015 Ending 6/30/2016
Johnston 3.25 months, Woodbury 3.25 months, Stafford 6.5 months
New
NIH 1R01EB021345-01 Beginning 1/1/2016 – 12/31/2017
Stafford 2.4 months
Ended
NSF MCB-1518528 Beginning 12/15/2014 Ending 11/30/2015
Woodbury .12 months

What other organizations were involved as partners?
PLCO, EEMS
Claire Zhu, Program Director
Early Detection Research Group
Division of Cancer Prevention
National Cancer Institute
9609 Medical Center Drive
Room 5E106
Rockville, MD 20850

Provided specimens for testing.
Duke University
Dr. Jeffrey Marks

Provided additional samples beyond the subaward requirements

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Altai State University

Russian-American Anticancer Center

61 Lenin St., Barnaul, Altai Territory, Russian Federation, 656049

Stephen Albert Johnston
Director, The Biodesign Institue
Center for Innovations in Medicine
Arizona State University

Stephen:

I am writing concerning my willingness to collaborate on your application of the immunosignature technology to breast cancer. I am the Director of the Russian American Anticancer Center in Altai. We established this Center explicitly to apply the IMS technology here in Russia. Our Core exactly replicates the one in CIM.

As part of our collaboration we initiated a prospective collection of samples from women with breast cancer, diagnosed at the Altai Regional Cancer Center. From this study we will be able to analyze the Stage 1 breast cancer sample you propose in your DoD grant. Unfortunately, we cannot for legal constraints send these samples to CIM. We can, however, process the CIM samples here as you propose.

I look forward to completing this work.

Sincerely,

05.10.16

Andrei I. Chapoval, Ph.D.
Director, Russian-American Anticancer Center
Altai State University
61 Lenin St., Barnaul
Altai Terretory, Russian Federation
656049
Phone: +7(3852)-298-142
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DURHAM, NORTH CAROLINA 27710

DEPARTMENT OF SURGERY
BOX 3873
email-marks003@mc.duke.edu

TEL. (919) 684-6133
FAX (919) 681-6291

9 May 2016

Stephen Albert Johnston
Director, The Biodesign Institue
Center for Innovations in Medicine
Arizona State University

Stephen:

I am writing to commit to my continued collaboration with you to apply the immunosignature diagnostic technology to breast cancer detection. I am acutely aware that this area is in dire need of useful biomarkers. Your approach is the most likely to result in a robust signature of the disease, even within the complex and heterogeneous environment of the disease and human variation.

I am disappointed to see that the PLCO samples are not of sufficient quality for the studies we planned. However, I strongly support the new focus on establishing Stage 1 and DCIS signatures. We have more than sufficient numbers of samples for the studies you propose on both conditions. As you know we have already done a pilot study on both types to demonstrate the performance of the samples. I am committed to providing you with as many of our high quality specimens as required to advance your discovery and valdition efforts.

I look forward to seeing the results of this effort and continue to work with you in this critical undertaking.

Sincerely,

Jeffrey Robert Marks

Digitally signed by Jeffrey Robert Marks
DN: cn=Jeffrey Robert Marks, o=Duke University, ou=Department of Surgery, email=marks003@mc.duke.edu, c=US
Date: 2016.05.09 10:15:37 -04'00'

Jeffrey R. Marks, Ph.D.
Department of Surgery
Duke University

BIOPRESERVATION AND BIOBANKING
Volume 12, Number 6, 2014

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BRIEF REPORT

Laboratory Detective Work Identifies a Mishandling Problem in Sample Aliquoting

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Data from a recent ovarian cancer biomarker study using serum aliquots from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial Biorepository showed that CA125II concentrations in these aliquots were significantly lower than those previously measured in the same subjects from the same blood draw. We designed an experiment to investigate whether samples used in the study (reference study) were compromised during the aliquoting process. We measured CA125II in the ''sister'' vials created during the same aliquoting process as the reference study aliquot, and in ''cousin'' vials newly aliquoted from another parent vial from the same blood draw, from 15 healthy controls in the study. Because the sister vials were created in a specific order, we also assessed whether there was a CA125II concentration gradient among the sisters. The Wilcoxon signed-rank test was used to test the statistical significance of the observed differences. Mean CA125II concentration (volume-averaged) was greater in the sisters than the cousins in all 15 subjects ($p < 0.001$). The mean coefficient of variation was 0.25 (range: 0.12–0.43) in the sisters and 0.11 (range: 0.–1.1) in the cousins ($p < 0.008$). The mean ratio of CA125II in the 5th aliquoted versus the 3rd aliquoted sister vial was 1.66 (1.25–2.5, $p < 0.001$). These data suggest that the parent vials were not adequately mixed before they were aliquoted. CA125II in serum can partially precipitate to form a concentration gradient in long-term storage. Rigorous vortexing after thawing and before aliquoting is thus critical.

Introduction

The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial Biorepository stores over 2.8 million specimens including serum, plasma, buffy coat, and red blood cells.[1] Since 2005, this resource has been utilized in over two hundred published studies.[2]

In 2009, a PLCO longitudinal study of several ovarian cancer biomarkers (including CA125) was initiated by Fred Hutchinson Cancer Research Center (FHCRC) investigators. Approximately 4500 serum vials from ovarian cancer cases and controls, all previously untouched ''parent'' vials, were pulled from the PLCO Biorepository and sent to the processing lab for aliquoting in 2011. Aliquots of 0.3 mL were made for the study at the processing lab and shipped to FHCRC.

In 2013, NCI was contacted by Dr. Nicole Urban, the PI of the above-mentioned study (''reference study''), who noted that the CA125II levels measured in the samples at her FHCRC lab using a clinical assay (Abbott Labs) were much lower ($\sim$40%) than CA125II levels measured earlier as part of the ongoing PLCO trial for the same subjects and blood draw using a different clinical assay (Centocor). Additionally, CA125II levels in a set of PLCO samples aliquoted at the processing lab several years earlier for a different project but measured at FHCRC alongside the reference study samples using the same Abbott assay were very close to the CA125II levels (for the same subjects and blood draw) measured earlier for the ongoing trial.[5]

Communication records between the shipper at the processing lab and recipients at FHCRC demonstrated that there was no problem with shipping; communications between Biorepository personnel and the processing lab also did not indicate issues with the transit of the samples. Taken together, these observations appear to rule out sample degradation during transit or in the PI lab, and thus point to possible problems at the aliquoting at the processing lab. Due to a personnel change at the processing lab, the actual aliquoting protocol used at the time for the reference study samples could not be verified. We suspected that one of two possible scenarios may have occurred during aliquoting: 1) a prolonged sojourn at room temperature, or 2) a failure to adequately mix the parent vials (usually by vortexing) prior to the aliquots being taken.

Here we report the results of an experiment designed to determine which (if any) of the above scenarios likely occurred.

Materials and Methods

The experimental design takes advantage of several features of PLCO sample aliquots. In PLCO, when a parent vial ($\sim$1.8 mL) is first aliquoted for a study, the whole vial is aliquoted into a series of daughter vials according to a standard scheme specifying the aliquot volume and ordering.

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For a study sample volume of 0.3 mL, the processing lab aliquots the parent vial into daughter (sister) vials of the following volumes and in the following order: 0.3 mL (the study "daughter" aliquot), 0.2 mL, 0.3 mL, 0.15 mL, 0.5 mL, 0.2 mL, and 0.15 mL (all "sister" aliquots). Additionally, the PLCO biorepository retained an "aunt" vial, namely, an untouched second parent vial processed from the same vacutainer, for all reference study samples.

Fifteen reference study subjects were randomly selected for the experiment. For each, the "aunt" vial was aliquoted freshly to create "cousin" vials according to the standard protocol. Briefly, vials were thawed in 2–8°C overnight, mixed thoroughly by vortexing, and aliquoted into 0.3 mL "cousin" vials on ice within 24 hours. CA125II was measured in the sisters and two of the cousins using a CLIA assay (Elecsys CA 125 II assay, Roche). Because this assay required a minimum volume of 0.25 mL, sister vials with volume under 0.25 mL were pooled for CA125II measurements. All laboratory procedures were performed at PPD, Inc.

Statistical analysis

Mean (volume-averaged) CA125II concentration in the sisters and cousins were computed for each subject. The coefficient of variation (CV) for sisters and cousins within each subject was calculated as the standard deviation across sisters (cousins) divided by the mean of the sisters (cousins). To assess whether a concentration gradient existed among sisters, the ratio of the CA125II concentration in the 5th versus 3rd sister vial was computed (the other sister vials were pooled). The Wilcoxon signed-rank test was used to test whether the within-subject mean of CA125II level and the CV were different between the sisters and cousins and whether the CA125II level differed between the 5th and 3rd sisters.

Results

Two possible results were anticipated as follows: 1) Mean CA125II levels in sister vials are lower than in cousin vials and the CVs are similar, suggesting uniform sample degradation occurred during the aliquoting process, or 2) The mean CA125II level is higher in sisters than cousins, the CV is higher in sisters than cousins, and sister vials aliquotted later in the order have higher concentrations than those aliquoted earlier, suggesting that the parent vial was not mixed adequately before aliquoting.

Figure 1 shows the CA125II concentrations in the sister and cousin vials for all 15 subjects. The mean concentration was greater in the sisters than cousins in all subjects (range for difference in means: 0.37–5.3 U/mL; $p < 0.001$). The mean CV for the sister vials was 0.25 (range 0.12–0.43) compared to 0.11 (range 0–1.1) for the cousins ($p < 0.008$). The mean of the ratio of CA125II concentrations in the 5th versus 3rd sister vial was 1.66, range 1.25–2.5 ($p < 0.001$). The pooled sister vial, which contained the 2nd, 4th, 6th, and 7th ordered sister vial, generally had a CA125II concentration between that of the 3rd and 5th sister vials.

Discussion

In this report, we detailed our investigation to pinpoint a suspected laboratory error in the processing of serum aliquots. Our experimental data strongly suggest inadequate mixing of parent vials prior to aliquoting, resulting in a concentration gradient among the daughter (sister) vials. It is not surprising that a large molecule such as CA125 partially precipitated to form a concentration gradient during long-term storage. Thus, mixing parent vials vigorously before aliquoting is necessary.

Several lessons can be learned from this experience. Aside from the obvious importance of having a best practice Standard Operating Procedure (SOP), some quality control measures should be instituted for sample handling procedures. This can involve "spot-checking" by measuring standard markers in a small subset of the derivative samples after each process. A Laboratory Information Management System (LIMS) that records and tracks all aspects of the sample processing jobs is also essential.

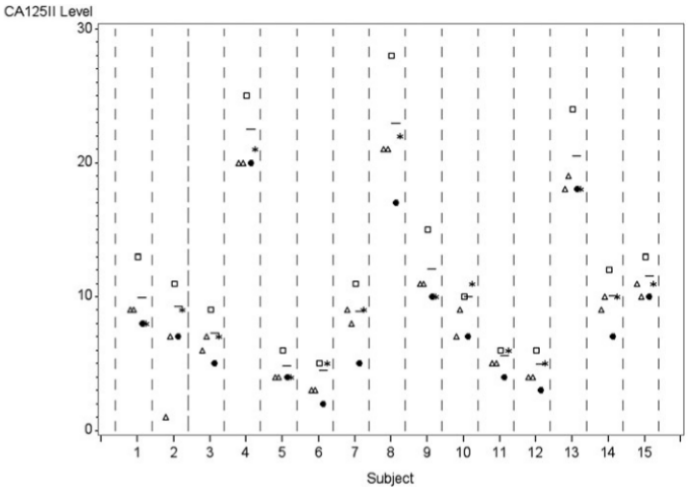

FIG. 1. CA125II concentrations in sister and cousin vials for all 15 subjects. *Triangles* (to the *left* for each subject) represent the two cousin vials. Sister vials (to the *right* for each subject) are represented as follows: *square* (5th sister vial), *dot* (3rd sister vial), *star* (pooled sister vials); *horizontal line* is (volume weighted) mean concentration among sisters.

Acknowledgment

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Author Disclosure Statement

No competing financial interests exist. 1058×626

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Stephen Albert Johnston

Director, The Biodesign Center
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12th May 2016

Dear Stephen,

Thank you for our discussions on the availability of the breast cancer samples from the UKCTOCS repository to which Abcodia has exclusive commercial access. These samples are of the highest quality and were used to validate the ROCA Test for ovarian cancer screening that Abcodia has commercialized. As you have reported, these samples also were useful for determining the pre-diagnostic immunosignatures of melanoma.

You have requested samples for your breast cancer study of: 1) samples 0-1 year prior to diagnosis of stage 1 breast cancer, 2) samples taken prospectively 1 to 2 years before diagnosis of stage 1 breast cancer and 3) samples taken 0-6 months prior to diagnosis of DCIS. Disease samples will be provided with the required relevant demographic and clinical information that is available to Abcodia.

We will also supply the required numbers of control samples. Subject to agreement on contractual terms and approvals of the Abcodia-UCL Steering Committee and Research Ethics Committee we would be glad to provide these samples at the negotiated price.

We look forward to the results of this important study. Abcodia and HealthTell Inc have initiated discussions on possible cooperation and this study will be relevant to these discussions.

Kind regards,

Julie Barnes, PhD
Chief Scientific Officer
Abcodia Ltd